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11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.	12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The long-range goal of this project is to understand what molecular changes allow some mammals to achieve and survive hibernation so that these molecular mechanisms may be engineered to evoke similar hypometabolic states in humans. Earlier results supported the hypothesis that differential gene expression is significant for determining the hibernating phenotype. Thus, one objective of this work has been to identify gene products that are differentially expressed during hibernation in ground squirrels. This question was addressed at both the mRNA and protein levels, and led to the identification of numerous gene products with significantly altered expression between summer and hibernating ground squirrels. These proteins provide targets for future work with a goal of improving outcomes after ischemic or hypothermic insults. Our second objective was to determine the control mechanism(s) used to suppress protein synthesis in the liver during hibernation; this aspect of our study defined a novel regulatory mechanism acting through eIF4E as the likely cause of reduced translation in the liver during torpor. Furthermore, this mechanism provides an elegant solution to the need for a reversible process that permits the reactivation, or even hyperactivation, of translation observed during each of the numerous arousals that punctuate the hibernation season.

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C. Final Progress Report.

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(4) Statement of the problem studied. Hibernation in mammals leads to profound reductions of metabolic, heart and respiratory rates in addition to core body temperatures. These events are highly controlled and spontaneously reversible, yet little is known about the molecular events that underlie this hibernating phenotype. An understanding of these events could lead to rational development of safe hypometabolic and hypothermic effectors for human applications. An important approach to gain a detailed molecular understanding of hibernation is to isolate and identify gene products that show unique patterns of expression during hibernation. This strategy offers great promise and has been under-exploited. We chose liver for this work because of its key roles in metabolism and in the maintenance of homeostasis for the organism. These functions are likely targets for survival-enhancing modifications during the physiological extremes experienced by hibernators. Such modifications include elevated expression of gene products that play an adaptive role during hibernation. For example, our previous work identified $\alpha_2$macroglobulin as one protein that is upregulated during hibernation. α_2Macroglobulin is a broad spectrum protease inhibitor; its abundance and protease inhibitory activity are elevated during hibernation in addition to the amount of its corresponding mRNA. A likely critical function of $\alpha_2$macroglobulin in hibernation is to reduce the rate of blood clotting. The first objective of the recently completed work was to isolate and identify additional differentially expressed gene products from the livers of hibernating animals. This was done by direct analysis of the liver proteome for differentially expressed or modified proteins. The second objective was to examine the presence and modification of critical regulatory factors that control the initiation of translation. Earlier results had demonstrated that translation reversibly slows during torpor, is reactivated during arousal and remains active throughout the interbout euthermic period that accompanies each bout of torpor. The goal of this work was to understand the molecular basis of this suppression and reversibility of protein synthesis for two reasons: First, as a means to understand whether hibernators are particularly adapted to continue to make gene products at low temperature, or whether they must return to higher temperatures in order to replenish gene products that are depleted during torpor. Second, the depression of protein synthesis during torpor then full restoration of activity during interbout arousal is a property shared by numerous other cellular processes; understanding the molecular mechanisms that control this reversibility may have broad implications for cell function and survival of a hypometabolic state.

(5) Summary of the most important results. The progress made during this funding period on each of our two Specific Objectives is described below:

Specific Objective 1. Identify differentially-expressed proteins in liver and blood. *Two-dimensional gel electrophoresis will be used to isolate proteins that are induced or uniquely modified during hibernation. These proteins will be identified using tandem mass spectrometry.*

Two studies on the liver proteome of golden-mantled grounds squirrels were completed during this funding period. The first was as proposed: we analyzed total liver proteins for differences among the torpid, interbout aroused and summer active states. In particular, since the animals are “protected” from cold and ischemic damage to tissues in winter, we expected to find proteins that confer this protected phenotype to be upregulated in both winter samples compared to summer. The second study was necessitated by the disappointing results of the first, which were that no liver proteins were found to be upregulated or uniquely modified in the winter samples relative to the summer. Elaine Epperson noted two features about the winter samples: first, there was much more individual variation in the protein patterns among individuals in the same winter state than in summer; second, the “quality” of the protein spots was diminished in the winter samples; i.e., they looked “fuzzy” compared to summer samples. Because the gels were always processed in sets of six, with liver extracts from two summer active, two winter active and two interbout aroused animals per set, the quality difference could not be due to procedural or gel differences, but rather must be due to some attribute of the

samples. Since we had earlier proposed that interbout arousals might be essential in order to restore gene products that are slowly lost during torpor, we wondered if the observed loss of quality of the samples on these 2D gels could be a reflection of exactly this process. We attempted to quantify the "fuzziness" and settled on a quantitative measurement called saliency that is determined by the 2D gel analysis software, Melanie4. The saliency is dramatically altered in both winter states compared to summer. A preliminary report of this finding was published in a conference proceedings (Martin et al., 2004), and we hope to pursue the molecular basis of this result in the future. Nevertheless, if our hypothesis is correct, namely that critical proteins are being slowly degraded/lost during torpor, then are restored during interbout arousal, it predicts that animals just re-entering torpor will have fully restored their complement of required proteins. Thus, the best samples to use in order to find and identify the crucial protein products for torpor would be obtained from animals re-entering torpor after an interbout arousal--hence the need for the second, unplanned study.

Liver extracts from nine entrance animals were compared to extracts from nine summer active animals. The percent volume for each spot was found using Melanie 4 software and the comparison made with a two-tailed Student's t-test, because, for almost all spots, it was not known whether to expect an increase or a decrease in winter. 961 groups (i.e. the same protein spot from all gels) were subjected to t-tests, and 130 were found to differ with a $p < 0.05$. Of these, 84 protein spots were found to be reproducibly resolved on enough gels for statistical analysis, in most cases this was all 18 gels. All gels had relatively high resolution throughout, enhancing quantitative assessment and comparison. Of the 84 reproducible and significantly changing groups, 28 had a higher protein steady-state level in summer, and 56 were higher in winter (entrance); that is, two-thirds of the proteins that changed were higher in entrance. Six spots gave no id, 10 spots gave multiple ids; thus we were unable to identify those proteins. We were pleased, however, that 68 proteins could be reliably identified using existing protein sequence databases from model organisms. This work has been published recently and provides both the encouragement to continue to examine changes in the proteome of liver and other organs that are associated with the hibernating phenotype as well as a number of hypotheses concerning the significance of the various altered proteins for hibernation (Epperson et al., 2004, see appendix).

Specific Objective 2. Analyses of the biochemical basis of translational depression during torpor*. The results of previous experiments demonstrated that translational depression during torpor is significant and likely achieved by a combination of temperature effects and active mechanisms. The biochemical basis for the active component will be sought by a systematic screen for differential expression and modification of key translation factors*.

We surveyed liver extracts for a battery of key translation factors, examining both their presence and their phosphorylation status. Extracts from summer active animals were compared to extracts from hibernators during either interbout arousal or torpor. The most significant finding was that eIF4E-BP1 is seasonally regulated, such that it is only expressed in winter. In addition, it is differentially phosphorylated as animals cycle between torpor and interbout arousal, suggesting a novel mechanism for depressing translation of capped mRNAs during torpor which can quickly be reversed to allow translation to proceed with maximum efficiency during each interbout arousal. These results were published recently in the American Journal of Physiology (van Breukelen et al., 2004, see appendix). This mechanism, together with findings from an earlier study to examine translational activity across hibernation using polysome profiles, raises the possibility that some proteins, specifically that do not rely on cap-dependent mechanisms, may be preferentially translated during early arousal. This finding is worthy of further studies and is pursued in a pending application.

For more detail on the results from work completed under both of these Specific Objectives, please see the appropriate publications.

(6) A. Manuscripts published in peer reviewed journals during this 3 year funding period:

van Breukelen, F. and Martin, S.L. (2001) Translational initiation is uncoupled from elongation at 18°C during mammalian hibernation, *Am. J. Physiol*. **281**, R1374-R1379.

Epperson, L.E. and Martin, S.L. (2002) Quantitative assessment of ground squirrel mRNA levels in multiple stages of hibernation, *Physiol. Genomics* **10**, 93-102.

van Breukelen, F. and Martin, S.L. (2002) Reversible depression of transcription during hibernation. *J. Comp. Physiol*. **172**, 355-361.

van Breukelen, F., Sonenberg, N. and Martin S.L. (2004) Seasonal and state dependent changes of eIF4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor, *Am J Physiol Regul Integr Comp Physiol*. **287**, R349-R353

Epperson, L.E., Dahl, T. and Martin, S.L. (2004) Quantitative analysis of liver protein expression during hibernation in the golden-mantled ground squirrel, *Mol. Cell. Proteomics.* **3**, 920-933.

van Breukelen, F. and Martin, S.L. (2002) Molecular adaptations in mammalian hibernators: unique adaptations or generalized responses? *J. App. Physiol*. **92**, 2640-2647.

Carey, H.V., Andrew, M.T. and Martin, S.L. (2003) Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature, *Physiological Reviews* 83, 1153-1181.

(6) B. Published conference proceedings (was peer-reviewed):

Martin, S.L., Dahl, T. and Epperson, L.E. (2004) Slow loss of protein integrity during torpor: a cause for arousal? in *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application*, Barnes B.M. and Carey H.V. eds., Institute of Arctic Biology, Fairbanks, AK, pp.199-208.

(7) Scientific personnel supported by this project:

Sandra L. Martin, Ph.D., PI

Frank van Breukelen, Ph.D, Post-doctoral trainee

L. Elaine Epperson, graduate student April 2001-July 2003, post-doc, August 2003-August 2004. Ph.D. awarded for work on this project August 2003.

(8) Report of inventions: N/A

(9) Bibliography

Epperson, L. E., Dahl, T., and Martin, S. L. (2004). Quantitative analysis of liver protein expression during hibernation in the golden-mantled ground squirrel. Mol Cell Proteomics *3*, 920-933.

Martin, S. L., Dahl, T., and Epperson, L. E. (2004). Slow loss of protein integrity during torpor: a cause for arousal? In Life in the Cold: Evolution, Mechanisms Adaptation, and Application, B. M. Barnes, and H. V. Carey, eds. (Institute of Arctic Biology, Fairbanks), pp. 199-208.

van Breukelen, F., Sonenberg, N., and Martin, S. L. (2004). Seasonal and state-dependent changes of eIF4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor. Am J Physiol Regul Integr Comp Physiol *287*, R349-R353.

(10) Appendixes: Please see reprints of all three of the above Bibliography articles as pdfs, each sent with a report documentation page . Please note that these are a critical part of the final report. This is because all of the work under these Specific Objectives is now published, so instead of providing detailed explanation and figures in this report, we provide appropriate reference to the completed, published work and the reprints.